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First report of "*Candidatus* Phytoplasma solani" on a new host marigold (*Tagetes erecta* L.)

Şevket ALP[1], Mustafa USTA[2], Hikmet Murat SİPAHİOĞLU[3,*], Abdullah GÜLLER[2]

[1]Department of Landscape Architecture, Faculty of Agriculture, Yüzüncü Yıl University, Van, Turkey
[2]Department of Plant Protection, Faculty of Agriculture, Yüzüncü Yıl University, Van, Turkey
[3]Department of Plant Protection, Faculty of Agriculture, Inönü University, Battalgazi, Malatya, Turkey

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Abstract: Marigold (*Tagetes erecta* L.) plants, also called Mexican or Aztec marigold, with symptoms of shoot proliferation, dwarfing, and reddening were observed in ornamental gardens of Van Province (Turkey). Five plants, two of them showing reddening and three symptomless plants, were sampled at the end of September 2014. Genomic DNA isolated from symptomatic and nonsymptomatic plant leaves was used to amplify 16S rDNA fragments by nested polymerase chain reaction (PCR). Of the 5 marigold samples tested by PCR, only the two showing reddening symptoms yielded the expected 1.2-kb DNA fragments. Amplified PCR fragments were cloned into a plasmid vector and transformed into competent *Escherichia coli* strain JM 109. Recombinant plasmid DNA was isolated and sequenced bidirectionally. The provided sequences were 1244 bp and 1245 bp in length and were designated as isolate 1 and isolate 2, respectively. BLAST analysis of the 16S rDNA sequence and virtual restriction fragment length polymorphism (RFLP) analysis confirmed the presence of the phytoplasma "*Candidatus* Phytoplasma solani". The in silico virtual RFLP pattern of isolate 1, based on the 16S rDNA F2n/R2 fragment, was identical (similarity coefficient 1.00) to the reference pattern of 16Sr group XII, subgroup A (GenBank accession no.: AF248959). Isolate 1 was identified as a member of 16SrXII-A. Based on the same analyses, isolate 2 showed molecular characteristics different from reference patterns of all previously established 16Sr groups and subgroups. The most similar was the reference pattern of 16Sr group XII, subgroup A (GenBank accession no.: AF248959), with a similarity coefficient of 0.97. This is the first report of naturally occurring "*Ca.* P. solani" affecting *T. erecta*, which shows that this plant species is an alternate host of the agent.

Key words: "*Candidatus* Phytoplasma solani", *Tagetes erecta*, nested PCR, molecular cloning

1. Introduction

Phytoplasmas are phloem-limited, insect-transmitted, wall-less, nonculturable plant pathogens from the class *Mollicutes*. They cause diseases in numerous plant species including fruit, vegetable, cereal, forest, and ornamental crops worldwide (Lee et al., 2000). Molecular methods and interactive online Web software have become the most reliable tools for the detection, identification, and classification of phytoplasma diseases. These methods are most commonly used to amplify either an entire or a specific phytoplasma sequence of 16S rDNA and to generate in silico digestions with a few key enzymes. The latter may help to distinguish the input data from previously recognized patterns (Lee et al., 1998; Khadhair et al., 2008).

Following the application of molecular technologies, phytoplasma taxonomy is largely or entirely based on analysis of 16S rRNA gene sequences. "*Candidatus* Phytoplasma solani" falls within the 16SrXII group containing phytoplasmas such as "*Ca.* P. japonicum", "*Ca.* P. fragariae", and "*Ca.* P. australiense", which infect a wide range of crop plants (Duduk and Bertaccini, 2011; EFSA, 2014).

An important ornamental plant, marigold (*Tagetes erecta* L.) is grown in homes and gardens throughout Turkey. In addition to their ornamental role (Wright, 1979), marigold plants have been used as pharmaceutical plants (Tostle, 1968) and pesticides for the protection of agricultural crops (Morallo and Decena, 1982; Kourany and Arnason, 1988; Rhoades, 1990). Although "*Ca.* P. solani" has been known on wild marigold, *Calendula officinalis* (common marigold) (Esmailzadeh-Hosseini et al., 2011), the presence of the disease on *T. erecta* (marigold) has not been reported. Today, the only phytoplasma disease reported on *T. erecta* is marigold phyllody, which belongs to the aster yellows group (16SrI), subgroup B (Almeyda-León and Rocha-Peña, 2001; Rojas-Martínez et al., 2003).

* Correspondence: murat.sipahioglu@inonu.edu.tr

"*Candidatus* Phytoplasma solani" (Quaglino et al., 2013), formerly known as Stolbur phytoplasma (taxonomic group 16SrXII-A), affects a wide range of wild and cultivated plants (Marcone et al., 1997). In Turkey, "*Ca*. P. solani" has been detected in solanaceous crops (Sertkaya et al., 2007; Özdemir et al., 2009; Çağlar et al., 2010) and in pomegranates (Gazel et al., 2016).

In this study, we describe the identification of "*Ca*. Phytoplasma solani" associated with marigold reddening and the first report of "*Ca*. Phytoplasma solani" occurring in marigold, both in Turkey and worldwide.

2. Materials and methods

2.1. Plant material and DNA extraction

Leaves of naturally infected *Tagetes erecta* plants showing the symptoms of shoot proliferation, dwarfing, and reddening were collected. A total of five plant samples were collected from leaves of two symptomatic and three nonsymptomatic marigold plants at the end of September 2014 from an ornamental garden in Van Province (Turkey). Total genomic DNA was extracted from fresh leaf tissues by using a commercial DNA extraction kit (Vivantis Technologies, Oceanside, CA, USA). An isolate of Stolbur phytoplasma, identified from preliminary tests of plants from the same garden-grown marigold, was used as a positive source for diagnosis of the agent. The infected plant was maintained in a pot in a growth chamber and served as a phytoplasma source during the trials. DNA from a healthy marigold plant was used as a negative control.

2.2. Detection of "*Ca*. Phytoplasma solani"

Two universal phytoplasma nested primer sets (R16mF2/R16mR1 and R16F2n/R16R2) designed for amplification of phytoplasma 16S rDNA (Lee et al., 1993; Gundersen and Lee, 1996) were employed to detect phytoplasma DNA in samples prepared from fresh marigold leaf tissues. Nested polymerase chain reaction (PCR) was performed in an Eppendorf Mastercycler thermal cycler (Hamburg, Germany). A reaction volume of 50 µL contained PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.25 µL of each primer, 1 µL of sample DNA, and 0.5 U of GoTaq Green polymerase (Promega, Madison, WI, USA). The reaction program was 2 min for an initial denaturation step at 94 °C followed by 1 min of denaturation at 94 °C, annealing for 2 min at 55 °C, and extension for 3 min at 72 °C for 35 cycles with a final extension at 72 °C for 10 min (Lee et al., 1993). PCR products were separated by 1% agarose gel electrophoresis, stained with ethidium bromide, and visualized with a UV trans-illuminator.

2.3. Cloning and sequencing

Amplicons from the PCR obtained by nested primer sets were cloned and sequenced bidirectionally. For cloning, purified DNA fragments were ligated to the pGEM-T Easy Vector (Promega) and transformed into JM109 competent cells by electroporation. Recombinant plasmid DNA containing insert DNA was isolated and purified with a commercial Miniprep kit (Fermentas, Vilnius, Lithuania).

2.4. Sequence retrieval, alignment, and cladistic analysis

The sequences of phytoplasma 16S rDNA were retrieved online from the National Center for Biotechnology Information (NCBI) nucleotide sequence database (http://www.ncbi.nlm.nih.gov/gquery/gquery.fcgi) and compared with homologous DNA sequences available from the NCBI. The 16S rDNA sequences of the phytoplasmas studied in this work along with 22 other phytoplasmas representing distinct phytoplasma groups are shown in the Table and were aligned using the neighbor-joining method of MEGA 4.0 (Tamura et al., 2007). Relationships were assessed using 1000 bootstrap replicates.

2.5. In silico restriction enzyme digestions, virtual gel plotting, comparison of virtual RFLP patterns, and calculation of similarity coefficients

Virtual restriction fragment length polymorphism (RFLP) patterns were obtained from the trimmed sequences of 16S rDNA by exporting the Web-based virtual gel plotting program i*Phy*Classifier software (Wei et al., 2007). Each 16S rDNA fragment was digested in silico with 17 distinct restriction enzymes: *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI (*Tha*I), *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI (*Mbo*I), *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I. These enzymes were used for phytoplasma 16S rDNA RFLP analysis (Lee et al., 1998). Following in silico restriction digestion, a virtual 1.0% agarose gel electrophoresis image was plotted automatically to the computer screen. The virtual gel image was then captured for subsequent RFLP pattern comparisons. A similarity coefficient was calculated using Web-based i*Phy*Classifier software (Wei et al., 2007).

3. Results

3.1. Detection and identification of "*Ca.* P. solani" using universal primers

Phytoplasmas associated with reddening of leaves, shoot proliferation, and dwarfing of marigold plants (Figures 1a and 1b) in Van Province (Turkey) were detected by nested PCR with two universal primer pairs (R16mF2/R16mR1 and R16F2n/R16R2) in 2 out of 5 marigold samples. Typical bands of 1.2 kb were visualized in agarose gel specific to phytoplasma from marigold and the positive control, as shown in Figure 2. No amplicon was observed in the negative control when DNA from asymptomatic plants was used as a template.

DNA sequence comparisons of 16S rDNA of phytoplasma isolates (isolate 1 and isolate 2; sequences deposited in GenBank) revealed 99% sequence identity with "*Ca*. P.

Table. List of 16S rRNA gene sequences of phytoplasma strains used to compare "*Ca*. Phytoplasma" species. Phytoplasma isolates for which nucleotide sequences were determined in this study are indicated in bold.

16Sr group	Strain	GenBank accession no.	Reference
16SrIII: X-disease group			
III-A	Western X-disease phytoplasma	L04682	1999 (GenBank submission)
16SrV: elm yellows group			
V-A	"*Ca.* Phytoplasma ulmi"	AY197655	Lee et al. (2004b)
16SrVI: clover proliferation group			
VI-A	"*Ca.* Phytoplasma trifolii"	AY390261	Hiruki and Wang (2004)
16SrVII: ash yellows group			
VII-A	"*Ca.* Phytoplasma fraxini"	AF092209	Griffiths et al. (1999)
16SrX: apple proliferation group			
X-A	"*Ca.* Phytoplasma mali"	AJ542541	Seemüller and Schneider (2004)
16SrXI: rice yellow dwarf group			
XI-A	"*Ca.* Phytoplasma oryzae"	AB052873	Jung et al. (2003b)
16SrXII: Stolbur group			
XII-A	"*Ca.* Phytoplasma solani"	AJ964960	Firrao et al. (2005)
XII-A	"*Ca.* Phytoplasma solani" STOL	AF248959	
XII-D	"*Ca.* Phytoplasma japonicum"	AB010425	Sawayanagi et al. (1999)
	"*Ca.* Phytoplasma solani" isolate 1	KJ957010	This publication
	"*Ca.* Phytoplasma solani" isolate 2	KJ957011	This publication
16SrXIV: Bermuda grass whiteleaf group			
XIV-A	"*Ca.* Phytoplasma cynodontis"	AJ550984	Marcone et al. (2004b)
16SrXV: hibiscus witches' broom group			
XV-A	"*Ca.* Phytoplasma brasiliense"	AF147708	Montano et al. (2001)
16SrXVI: sugarcane yellow leaf syndrome group			
XVI-A	"*Ca.* Phytoplasma graminis"	AY725228	Arocha et al. (2005)
16SrXVII: papaya bunchy top group			
XVII-A	"*Ca.* Phytoplasma caricae"	AY725234	Arocha et al. (2005)
16SrXVIII: American (TX+NE) potato purple top wilt group			
XVIII-A	"*Ca.* Phytoplasma americanum"	DQ174122	Lee et al. (2006)
16SrXIX: Japanese chestnut witches' broom group			
XIX-A	"*Ca.* Phytoplasma castaneae"	AB054986	Jung et al. (2002)
16SrXX: buckthorn witches' broom group			
XX-A	"*Ca.* Phytoplasma rhamni"	X76431	Marcone et al. (2004a)
16SrXXI: pine shoot proliferation group			
XXI-A	"*Ca.* Phytoplasma pini"	AJ632155	Schneider et al. (2005)
16SrXXII: Nigerian coconut lethal decline (LDN) group			
XXII-A	Phytoplasma sp. strain LDN	Y14175	Tymon et al. (1998)
16SrXXVI: Mauritius sugarcane yellows D3T1 group			
XXVI-A	Sugarcane phytoplasma D3T1	AJ539179	2003 (GenBank submission)
16SrXXVII: Mauritius sugarcane yellows D3T2 group			
XXVII-A	Sugarcane phytoplasma D3T2	AJ539180	2003 (GenBank submission)
16SrXXVIII: Havana derbid phytoplasma group			
XXVIII-A	Derbid phytoplasma	AY744945	2004 (GenBank submission)
Outgroup			
A. laidlawii 16S ribosomal RNA	*Acholeplasma laidlawii*	M23932	

Figure 1. Reddening (a) and apical shoot proliferation (b) symptoms observed on infected Mexican (Aztec) marigold plants.

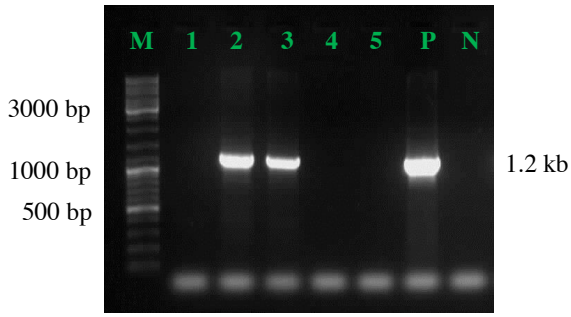

Figure 2. Detection of "*Ca*. Phytoplasma solani" by nested polymerase chain reaction (PCR) using universal primer pairs. Lanes 1–5 are tested samples, lane 2 (1.2 kb) and lane 3 (1.2 kb) are positively reacted samples; P: positive control, N: negative control, M: 10,000 bp molecular markers.

solani" (GenBank accession no.: AF248959), ribosomal group XII. BLAST searches of the sequences of isolate 1 and isolate 2 yielded best hits with "*Ca*. P. solani", subgroup XII-A.

3.2. Virtual RFLP and cladistic analysis

Virtual PCR-RFLP analyses of DNA sequences of marigold phytoplasma isolate 1 (GenBank accession no.: KJ957010) with 17 restriction enzymes [*Alu*I, *Bam*HI, *Bfa*I, *Bst*UI (*Tha*I), *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI (*Mbo*I), *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I] gave identical restriction patterns (similarity coefficient 1.00) to the reference pattern of phytoplasma STOL (GenBank accession no.: AF248959) (Figure 3). However, the same analyses of marigold phytoplasma isolate 2 (GenBank accession no.: KJ957011) (Figure 3) revealed differences with the reference phytoplasma STOL (GenBank accession no.: AF248959), as well as the reference patterns of all previously established 16Sr groups and subgroups. Isolate 2 exhibited unique RFLP patterns with the restriction endonucleases *Hae*III that clearly differentiated it from isolate 1 and 16SrXII phytoplasma subgroups. The RFLP profile obtained from isolate 2 phytoplasma amplicon is shown in Figure 3. Similarity coefficients derived from virtual RFLP analysis of the R16F2n/R2 16S rDNA sequence of isolate 2 phytoplasma were compared with those of 16S rDNA sequences of selected 16SrXII phytoplasma subgroups. The similarity coefficient values for isolate 2 were equal to 0.97, the threshold similarity coefficient for delineation of a new subgroup RFLP pattern type within a given group (Wei et al., 2007). With the present findings, isolate 2 should be a new subgroup under the SrXII group or should be a variant (A*) of subgroup 16SrXII-A.

A phylogenetic tree constructed by maximum parsimony analyses of 16S rDNA sequences of two Turkish marigold phytoplasma isolates and 22 other phytoplasmas from GenBank confirming that isolate 1 is most closely related to 16SrXII and subgroup A is given in Figure 4 (IRPCM Phytoplasma/Spiroplasma Working Team-Phytoplasma Taxonomy Group, 2004). Therefore, it is proven that "*Ca*. P. solani" isolate 1 caused disease on marigold plants in the symptomatic garden.

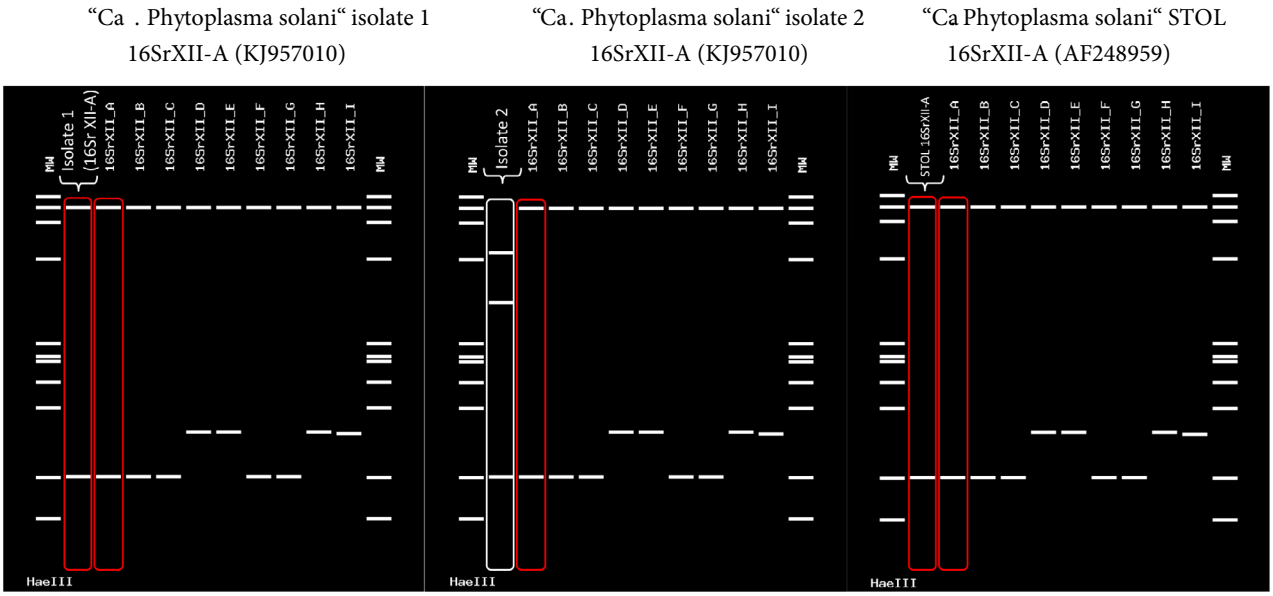

Figure 3. Virtual R16F2n/R2 RFLP analysis by key enzyme *Hae*III showed a clear separation into two distinct patterns of isolates 1 and 2, distinguishing "*Ca.* P. solani" isolate 2 (white box) from isolate 1 and "*Ca.* P. solani" STOL16SrXII, a reference strain (red boxes at same sites). The subgroup of isolate 2 was not included because it might be a new subgroup. Virtual RFLP analysis indicated that these two isolates were not identical: MW, FX174DNA.

4. Discussion

The phytoplasma detection assays confirmed the presence of phytoplasma disease in marigold plants collected from Van Province (Turkey). Previously, it seemed that "*Ca.* Phytoplasma asteris" (causal agent of marigold phyllody), which was the only phytoplasma detected on marigold to date, caused disease on marigold plants. However, sequence analyses and virtual RFLP of marigold phytoplasma isolate 1 clearly indicated that "*Ca.* Phytoplasma solani" was the causal agent of phytoplasma-like symptoms in marigolds.

Computer-simulated in silico restriction analyses were carried out with nearly full-length fragments of R16F2n/R16R2 of "*Ca.* P. solani" isolates (isolate 1 and isolate 2). With the exception of a digestion profile obtained by *Hae*III digestions, the virtual RFLP patterns of two isolates were identical for all of the other 16 key restriction enzymes. Virtual RFLP analysis generated distinct RFLP patterns between two isolates, indicating genetic diversity between two phytoplasma isolates. They were separated only by the *Hae*III digestion profiles given in Figure 3.

The availability of phytoplasma 16S rRNA gene RFLP pattern types (Lee et al., 1993b, 1998, 2000) has made possible the accurate and reliable identification, differentiation, and classification of phytoplasmas. Previously established phytoplasma 16S rRNA gene RFLP patterns have served as standard keys for phytoplasma strain identification and classification. Therefore, RFLP analysis still remains a useful tool for phytoplasma identification, differentiation, and classification (Wei et al., 2007). The virtual RFLP analysis method used in this study quickly generates reproducible RFLP patterns. These patterns reveal new pattern types that have not been recognized previously, providing additional standard keys for future identification and classification of the rapidly growing numbers of phytoplasmas (Wei et al., 2007).

These results clearly suggest that one of the phytoplasma isolates (isolate 1) studied in this work is a member of the 16SrXII group of phytoplasmas. The present results indicate that marigold is a new host for "*Ca.* P. solani" in Turkey. This new host of Stolbur phytoplasma could represent a new threat not only for marigolds but also for other solanaceous species grown in the same region. The emergence of this phytoplasma in the marigold represents ongoing evolution in the adaptation of "*Ca.* P. solani" to a new ecological niche (Arocha-Rosete et al., 2011).

Our results demonstrate for the first time that reddening, shoot proliferation, and apical dwarfing symptoms of marigolds are associated with "*Ca.* Phytoplasma solani". Further studies are needed to assess the taxonomic significance of isolate 2 in terms of sequence divergence and other properties to determine whether it belongs to a distinct "*Candidatus* Phytoplasma" subgroup

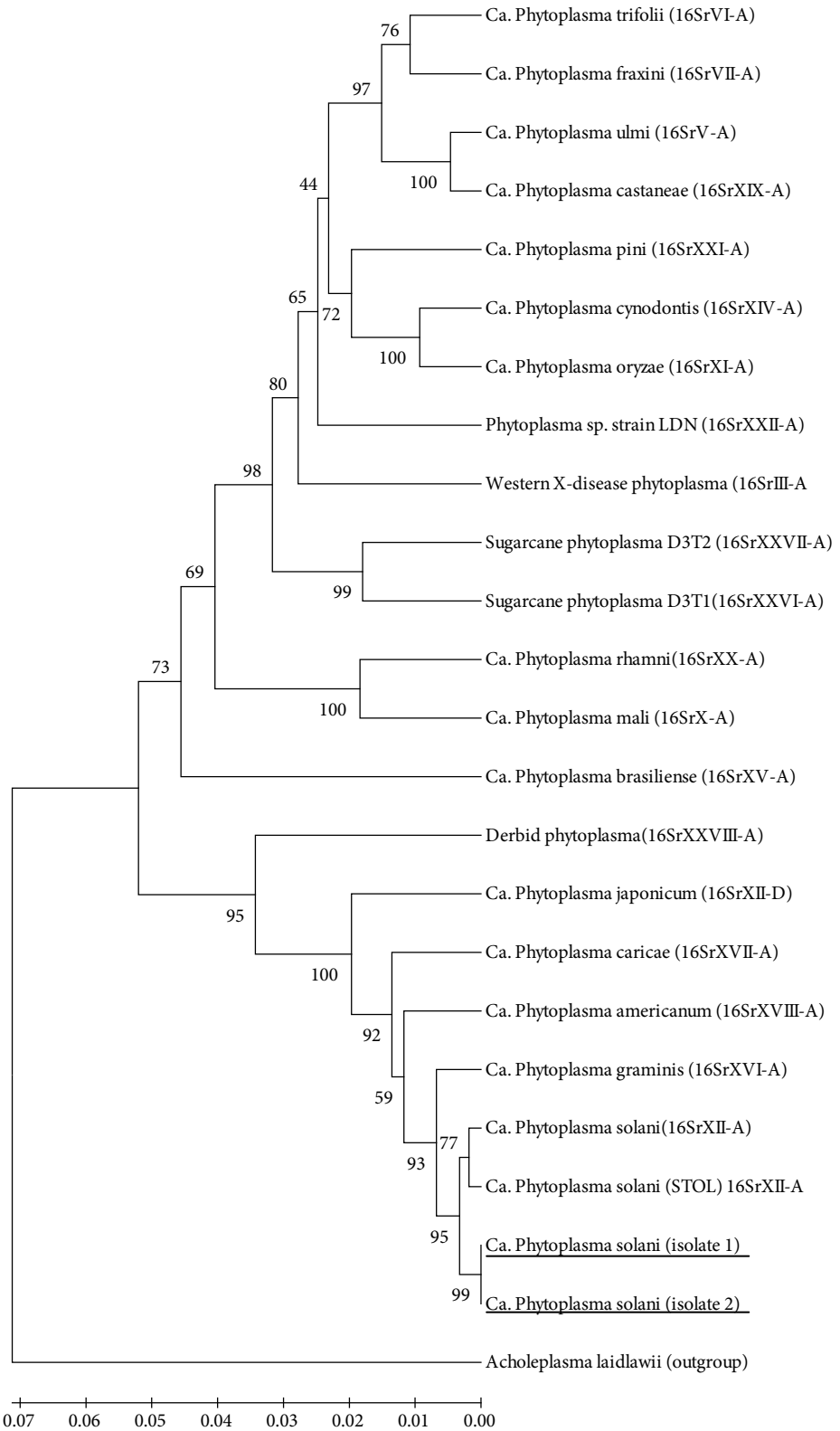

Figure 4. Phylogenetic tree constructed by maximum parsimony analysis of full-length 16S rDNA sequences from representative phytoplasma strains in the "*Candidatus* Phytoplasma solani" group (16SrXII) and other 16Sr phytoplasma groups. Sequences underlined were obtained from isolates used in this study. The reliability of the analysis was subjected to a bootstrap test with 1000 replicates. Bar: 0.01 nucleotide substitutions per site. *Acholeplasma laidlawii* was used as the outgroup to root the tree.

under 16Sr XII or is a variant (A*) of subgroup 16SrXII-A. Additional studies are needed to determine whether infected marigold plants can serve as an inoculum source for new infections to healthy plants established in flower gardens. Continued studies are also needed to determine the distribution, insect vectors, and economic impact of "*Ca.* Phytoplasma solani" on flower gardens.

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